

Cholesterol-Linked Pyrene Excimer Molecular Beacon with Enhanced Cell Permeability

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ABSTRACT

Covaleently labeled pyrene excimer molecular beacon (MB) with cholesterol moiety has been developed for enhanced the cellular delivery of MB.¹ Pyrene units were covalently attached into adenosine and incorporated to oligonucleotides at the complementary locations in opposite strands in the middle positions of hairpin stems. The system behaves as an effective MB that changes color from green to blue upon duplex formation. A cholesterol unit was also attached into a free terminus of one of these hairpins. The cholesterol-linked MBs enhanced the cellular delivery of the MBs and showed similar cell permeability to conventional transfection methods. These structurally simple cholesterol-based MB systems, which can be synthesized very efficiently, have good potential for opening up new and exciting opportunities in the field of in vivo biosensors.

INTRODUCTION

One of most powerful method for detection of the molecular dynamics of DNA and RNA in living cells is the molecular beacons (MBs).² Unfortunately, these systems are limited for use in vivo, because they are difficult to transfect into cells due to their polar anionic backbones and bulky structures. General transfection techniques, such as those employing liposomes or dendrimers, often result in false positive signals and significantly increase the aggregation signal in the endosomes.³ Electroporation and microinjection may cause severe damage to cells. To overcome these difficulties, we have developed novel cholesterol-linked MBs. The cholesterol unit positioned at the terminus of the hairpin MB allows these systems to enter into living cells efficiently without the need for any other transfection agent. In this symposium, we report such a system and demonstrate how it can be utilized effectively in vivo as well as other transfection agents.

RESULTS AND DISCUSSION

Pyrene-labeled nucleoside building blocks (A^{PY}) was synthesized through Sonogashira coupling of pyrene units to the 8-position of a 2'-deoxyadenosine base.⁴ We

incorporated A^{PY} into oligodeoxynucleotides (ODNs) by using the phosphoramidite method. Modified single-stranded ODN **1** containing two fluorophores in opposite strands of the stem of each hairpin and a perfectly matched sequence ODN **2** for hybridizing to the loop regions of ODN **1** were synthesized. In addition, cholesterol-bearing MB incorporating a cholesterol unit at the 5'-end of the hairpin (ODN **3**) was synthesized. Because the 5' and 3' termini of ODN **1** are unsubstituted, it is very easy to use an ODN synthesizer to modify them with functional molecules. The corresponding unmodified ODN **4** to compare the transfection properties between two systems and ODN **5** for hybridizing to the loop regions of ODN **3** and ODN **4** were also synthesized (Figure 1).

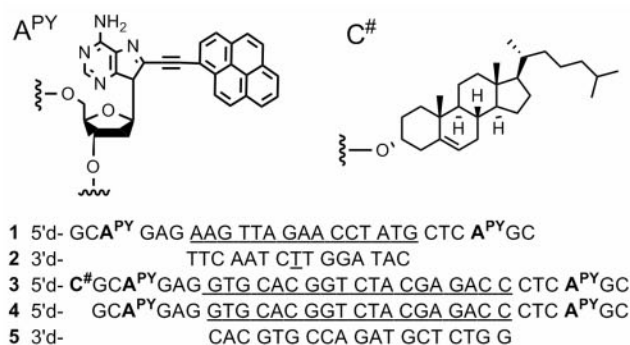


Figure 1. Molecular beacons and their target ODN sequences.

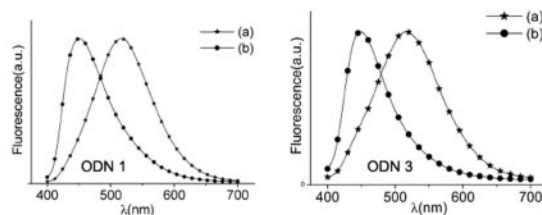


Figure 2. Fluorescence spectra of ODN 1 and ODN 3 in (a) duplex with target form and (b) hairpin state form

When pyrene-labeled MB ODN **1** is quenched in hairpin states, it emits a strongly red-shifted band at 521 nm (Figure 2). The fluorescence wavelength is very close to that of a typical pyrene excimer.⁵ In comparison, the value of maximum peak of the duplex between ODN **1** and ODN

2 is 450 nm. Even though we had attached a cholesterol moiety at the 5'-end of the MB, the photophysical signals of the MB system remained unaffected. We believe that the strongly red-shifted band at 509 nm must arise from stacking interactions between the two pyrene units of A^{PY}.

The cholesterol-bearing MB was successfully entered into living cells efficiently without the need for any transfection agent (Figure 3). The transfection efficiency of the MB conjugated with cholesterol (ODN 3) during the first 12 h in Huh7 cells was much higher than that of the unmodified MB (ODN 4). Unmodified MB showed essentially no signal even after 12 h. For the sake of comparison, the unmodified ODN 4 was transfected with a commercial transfection reagent, lipofectamine (ODN 4 + lipo). The fluorescence of transfection reagent case was similar to that of the cholesterol-linked MB.

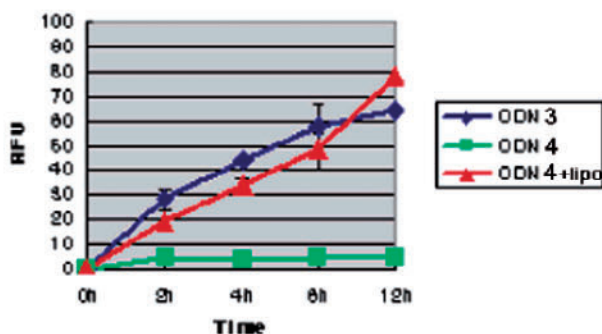


Figure 3. Time-dependent cellular uptake of ODNs 3 and 4 in the absence and presence of lipofectamine

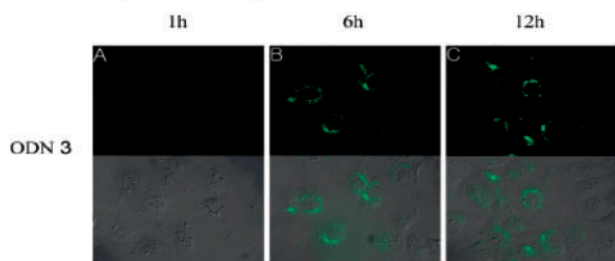


Figure 4. Cellular delivery and localization of cholesterol linked MBs in Huh7 cells.

To observe the transfection pattern of the cholesterol-bearing MB in Huh7 cells, a fluorescence microscopy experiment was performed (Figure 4). Consistent with our fluorometry experiments, these images clearly demonstrate that the cellular transfection of the ODN 3 (bearing the cholesterol unit) is far better than that of ODN 4 (without cholesterol) and that obtained by using the conventional transfection agent. Indeed, in the case of ODN 4, we observed no signal, as expected, whereas ODN 3 displayed extremely strong signals in the cytoplasm. To demonstrate whether the ODN 3 reached the cytoplasm of cells, double fluorescence staining experiments were carried out with LysoTracker.

Specially, the fluorescence signals obtained by using lipofectamine were concentrated in random “bright spots” in both the cytoplasm and the nucleus, indicating the aggregation pattern. We observed a similar trend after we transfected other oligonucleotides with lipofectamine in Huh7 cells. However, we did not observe any cross-signal or nonspecific aggregated bright spots in our MB system. Therefore, our MB system is more efficient than one using the transfection agent.

CONCLUSION

The pyrene excimer MB systems individually exhibit aromatic stacking between the opposing pyrene units in the stems. The systems can be used as effective MBs that change color from green to blue upon duplex formation. These novel MBs are relatively simple to synthesize, and their termini remain free for the introduction of other useful functionalities. In addition, we also introduced a cholesterol unit into one terminus of an MB and demonstrated clearly that the cellular delivery of this modified MB was enhanced significantly relative to those obtained by using the unmodified MB or by using conventional transfection agent-based methods. Cholesterol based transfection has the potential to avoid the endocentric pathway and to reduce the number of false positive signals arising from nuclease degradation. The cholesterol-bearing, pyrene-stacking-based MB system represents a new type of MB whose high cellular uptake efficiency makes such probes unique and novel. We believe that this one holds great promise for studying the dynamics of biomolecular systems in living cells and that its use will open up other new and exciting opportunities for biological applications in vivo.

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